

# **EXHIBIT 7**

# The Biology of Lymphocytic Choriomeningitis Infection: Virus-induced Immune Disease

JOHN HOTCHIN

*Division of Laboratories and Research, New York State Department of Health, Albany, New York*

Lymphocytic choriomeningitis, or LCM, is a naturally occurring virus disease of mice, from which the infection is occasionally transmitted to man. The virus has been known for nearly 30 years, since its first isolation by Armstrong and Lillie in 1934. The human disease varies in severity; it usually occurs as a mild respiratory infection which is sometimes followed by severe meningitis which can be prolonged and debilitating, occasionally fatal (Farmer and Janeway, 1942). Inoculation of the laboratory mouse with LCM virus causes a severe disease which is usually fatal, but natural infection of house mice and some laboratory mouse colonies takes the form of an inapparent latent infection. This paper concerns the results of a 5-year study of experimental murine LCM and the development of new concepts of the pathogenesis of animal virus disease. These concepts relate virus infection not only to immunological tolerance but also to several forms of acute immunological disease and, probably more important, to the autoimmune diseases occurring in later life; the intermediary events apparently have a bearing upon both the causation and prevention of virus-induced cancer.

As long ago as 1949 Burnet and Fenner made the suggestion that congenital LCM infection involved "the development of a tolerance to the foreign microorganism during embryonic life" which was in line with their hypothesis of a "self-marker" mechanism of immunological reactivity; this was based on the early work of Traub (1936a, 1938, 1939) on the continuous carriage of LCM virus in an infected mouse colony. This remark stimulated the present investigation into the mechanism of virus latency using LCM virus infection of mice as a model. It soon became evident (Hotchin and Cinitz, 1958; Hotchin, 1958) that this virus-host system did indeed involve a form of latency which did not depend on virus masking or on lysogeny but on the development of immunological tolerance to the virus by the host. Moreover, long-standing infection of a colony was not necessary since long-term tolerance with persistent infection could be induced by inoculation of the mice when only a few hours old. Before discussing the results concerning neonatal infection and the effects of various other agents, it will be helpful to consider the essentials of experimental infection in the adult mouse.

## THE BASIC LCM DISEASE PATTERN IN ADULT MICE

The LCM virus can cause an extremely wide spectrum of clinical and pathological effects in the albino mouse, ranging from inapparent, long-lasting infection through mild to severe illness with almost 100% mortality occurring about 8 days after inoculation. In our studies an LCM virus strain (designated UBC) derived from the Rockefeller Institute WE strain was used throughout except where stated and most of the experiments were performed with the Albany strain of albino mice. In the adult mouse, after intracerebral (ic) injection of 10 to 100 lethal doses, signs of disease appear after the fifth day with ruffling of the fur, a hunched posture, and blepharitis (Fig. 1). The signs become more severe, with weight loss, relative immobility and a "jumpy" reaction to loud noises; by the eighth day the animal is likely to go into fatal convulsions which can also be elicited by spinning it 10 to 20 times by the tail (Fig. 2). The usual post-mortem position of the animals is in the convulsion position with rear limbs extended, forelimbs flexed, neck extended, and thoracic spine flexed. This condition has been regarded as typical of LCM; it can also be caused by other agents including infection with some strains of mouse hepatitis virus (Gledhill, 1961) and anaphylactic shock. The sequence of events after inoculation by various routes is shown in Fig. 3. The pathology of LCM virus has been studied extensively by different workers, particularly Findlay and Stern (1936) and Lillie and Armstrong (1945), and the effects vary somewhat with different strains. In our hands the main findings were slight enlargement of the liver and spleen and commonly a yellow discoloration of the liver; histological examination showed well-marked lymphocytic meningoencephalitis and myelitis, severe hepatitis, with lymphocytic infiltration and foci of necrosis, interstitial pancreatitis, splenic hyperplasia, swollen hyperplastic lymph nodes, lymphocytic infiltration of salivary glands, testis, and occasional synovitis; there was also pneumonitis, cardiac valvulitis and pericarditis. The main histological feature of the disease is thus widespread lymphocytic infiltration. Virus multiplication occurs in all these organs as shown in our studies

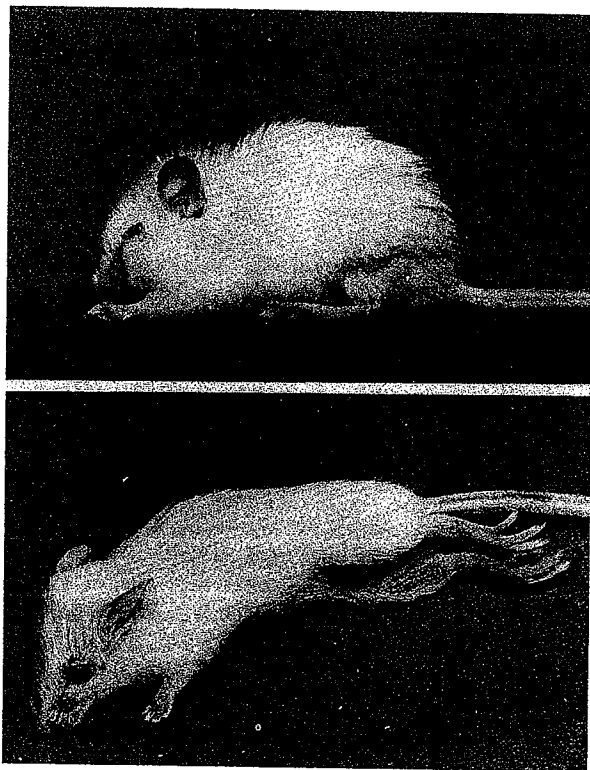


FIGURE 1. (Top) A mouse 6 days after ic inoculation with approximately 100 LD<sub>50</sub> LCM virus, showing ruffled fur, hunched posture, immobility, and blepharitis (facial edema).

FIGURE 2. (Bottom) Mouse during a "moderate" convulsion resulting from severe LCM disease, showing typically extended hind legs. The forelimbs had recovered at the time of the photograph.

and those of Rowe (1954) and Traub (1960). It is interesting to note that a similar list of organs of the monkey was reported by Armstrong, Wooley, and Onstott in 1936 as having titers of virus greater than that of circulating blood, suggesting that the disease pattern may be the same in the primates. This picture makes one feel that murine LCM should be called "lymphocytic choriohepatitis" or even "lymphocytic pan muritis." Larger doses of virus by the ic route cause a slightly earlier onset

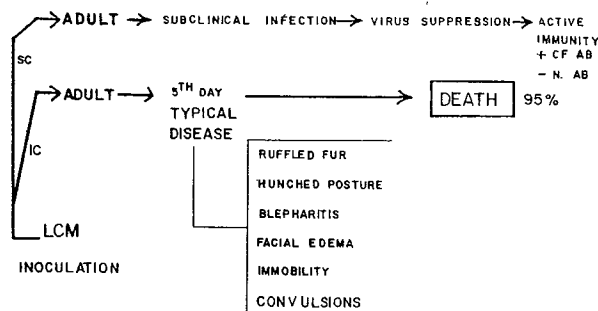


FIGURE 3. Diagram showing the pattern of extremes of LCM disease response in adult mice. sc = subcutaneous, ic = intracerebral.

of symptoms, and after very large doses there is often a puzzling decrease in severity and mortality of the disease; more will be said about this later. Subcutaneous (sc) or intranasal (in) inoculation of adults (see Fig. 3) causes only slight disease followed by complete immunity and the formation of complement-fixing but not appreciable neutralizing antibody; intraperitoneal (ip) inoculation causes intermediate effects between these extremes, varying with the viscerotropic or neurotropic tendencies of different virus strains. These patterns of disease were extensively studied and reported by Traub (1936a, b, c, 1938, 1939) and later much detailed work was described by Rowe in a relatively little known report (1954).

### THE PATTERN OF LCM DISEASE IN NEWBORN MICE

Traub (1936b) had shown that mice infected *in utero* with LCM virus carried the virus for many months, and this was confirmed by Haas (1941). Experimental inoculation of infant mice at different ages (Hotchin and Cinits, 1958; Hotchin and Weigand, 1961a) showed that there was a graded mortality with respect to age (Fig. 4); the newborn

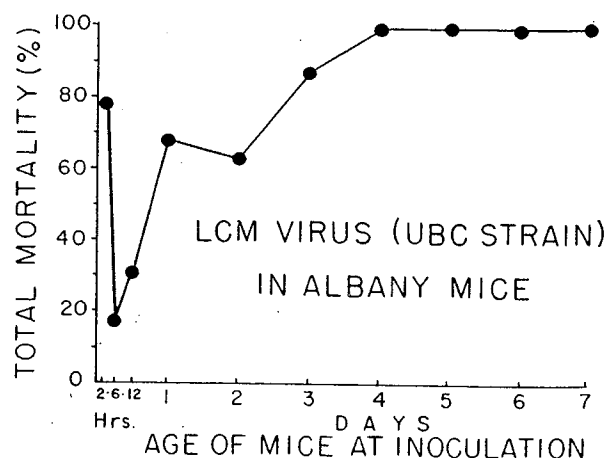


FIGURE 4. The relation between percentage mortality and age at inoculation with LCM virus. The 2, 6, 12, and 24 hr points are the average of three experiments.

mice were only slightly affected by ic doses of virus which approached 100% lethality for animals older than 4 days. The only sign of disease seen in the newborn was a temporary runting which was emphasized by comparison of the weight curves for control mice inoculated ic with normal mouse brain and for similar mice inoculated at 6 hours of age with LCM virus (Fig. 5); individual mice showed  $\frac{1}{3}$  to  $\frac{1}{2}$  the weight of controls, with loss of hair, weakness, blepharitis, and a "jumpy" reactivity (Fig. 6). A few runts died, the rest gained weight

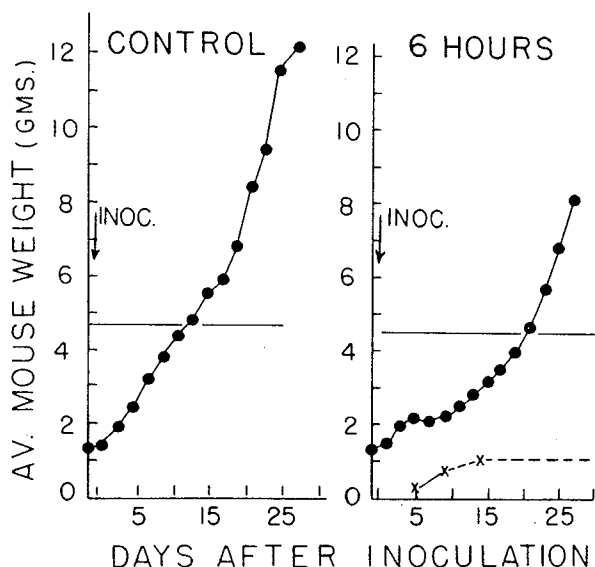


FIGURE 5. Weight and cumulative mortality of control mice inoculated ic with normal mouse brain and mice inoculated ic with LCM virus at 6 hr of age.

at the normal rate after the runt phase; as soon as 1 week after recovery they were almost indistinguishable from the controls (Fig. 7) and by the time they were adult they appeared normal in every

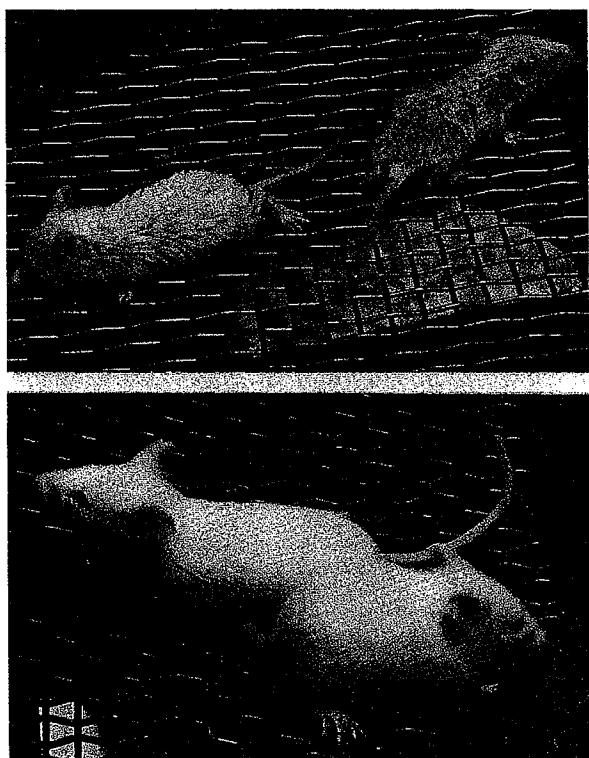


FIGURE 6. Runt (upper) and control (lower) mice. The runts had received neonatal ic LCM virus inoculation; their weight was  $\frac{1}{2}$  that of the controls and they showed loss of hair, weakness, blepharitis and jumpiness. Mice are all 4 weeks of age.

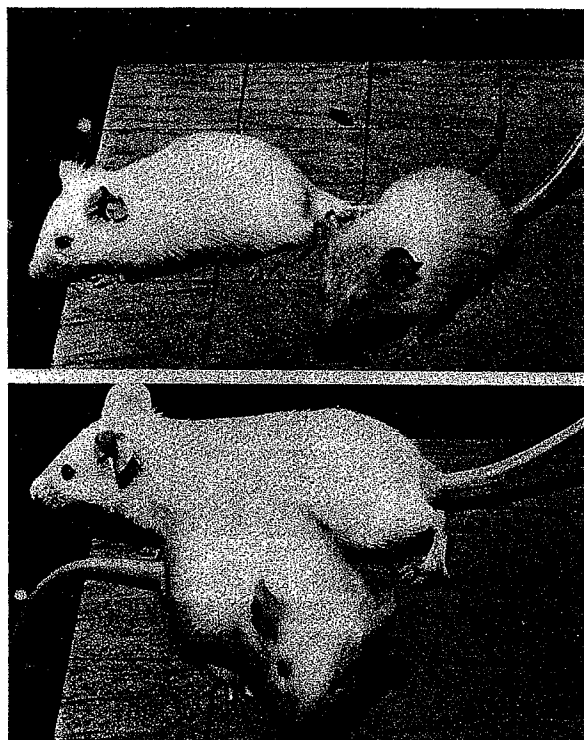


FIGURE 7. The same runt and control mice as in Fig. 6 photographed 1 week later showing marked recovery.

way; this sequence of events is summarized in Fig. 8. However, study of these recovered runts (Wiegand and Hotchin, 1961) showed that they were immune to LCM virus challenge and, more important, in spite of their normal appearance they carried high titers of virus in all organs and in their blood. This condition was named "Persistent Tolerant Infection" (PTI) since it resulted from the failure of the immune mechanism to rid the infant mouse of its infection, with the development of complete immunological tolerance toward the LCM virus antigen. Representative titers of the virus content of such PTI mice are shown in Table 1. The mice had no detectable complement-fixing nor neutralizing antibody in their blood but were immune to challenge.

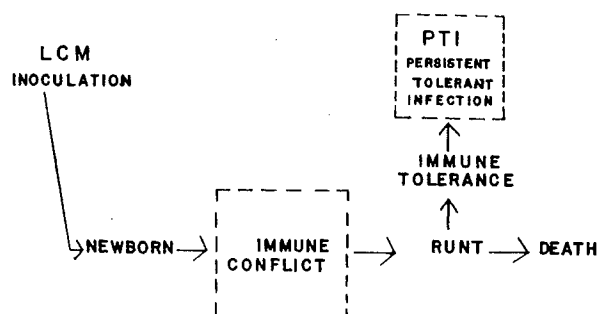


FIGURE 8. Diagram showing the pattern of LCM disease in newborn mice.

This immunity is clearly different from the active immunity of sc inoculated mice and was called "tolerant" immunity. The runt phase can be explained on the basis of a temporary and incomplete active response of the immature immune mechanism of the host attempting to eliminate the

TABLE 1. POOLED BRAIN AND POOLED BLOOD TITERS OF PTI MICE 195 DAYS AFTER INOCULATION

Age when inoculated with virus	LD <sub>50</sub> /g or ml	
	Brain	Blood
2 hr	10 <sup>5.8</sup>	10 <sup>4.67</sup>
12 hr	10 <sup>5.5</sup>	10 <sup>4.6</sup>
4 days	10 <sup>4.34</sup>	10 <sup>4.17</sup>
6 days	10 <sup>7.17</sup>	10 <sup>4.2</sup>

viral antigen and causing a temporary "immunological conflict" before it is overwhelmed by the increasing concentration of virus antigen; at this time it apparently succumbs to a negative feedback mechanism and complete immunological tolerance results. In the course of many experiments all mice tested within a few weeks of recovery from neonatal inoculation with LCM virus have proved to be PTI, carrying high titer virus in the blood. All appeared quite normal for 8 months or more, and the great majority continued to show high blood virus titers (10<sup>4</sup> LD<sub>50</sub>/ml or higher); they appeared to have normal birth rates and produced healthy litters of PTI animals; more will be said about them later.

#### FACTORS AFFECTING LCM DISEASE IN ADULT MICE

It was perfectly clear that LCM virus was innocuous to mouse cells, since mice tolerated high titers without any sign of disease and no cytopathic effect occurred after infection of primary mouse embryo tissue cultures of different types, or of several human cell lines. This has been shown by several workers (Eagle et al., 1956; Hotchin and Cinitis, 1958; Benson and Hotchin, 1960; Traub, 1962) in spite of continued multiplication of both cells and virus, although Ackermann (1960) has reported development of a mouse cytopathic LCM strain after repeated passage in vitro. Why then should the virus be able under certain circumstances to cause severe disease? It seemed likely that this was largely, if not wholly, explainable by the reverse of immunological tolerance, i.e., the interaction between the "harmless" virus and the immune response of the host. Since antibody appeared to play a small part in the suppression of virus (Traub, 1936a; Rowe, 1954), the effective part of the immune response appeared to be cellular, correlating with the extensive lymphocytic infiltra-

tion of the disease. Rowe (1956) reported a protective effect of X-rays upon mice infected with this virus and Haas and Stewart (1956) described a similar effect for amethopterin. Both of these agents inhibit leucocyte production and immune response in mice; their effects upon the course of LCM were therefore studied with other relevant agents with respect to the concept that the disease is due to an immunological conflict.

#### THE EFFECT OF X-RAYS

In this study (Hotchin and Cinitis, 1958; Hotchin and Weigand, 1961b) 2 different strains of both virus and host were compared; the general effect was the same in all cases and only 1 typical combination will be presented here. The results showed that pretreatment with X-rays caused well-marked protection of mice inoculated ic with LCM virus 24 hours later; the protection increased as dosage was raised to 500 r. After this point, the fully disease-protected animals died from the lethal effects of the irradiation (Fig. 9). The protection

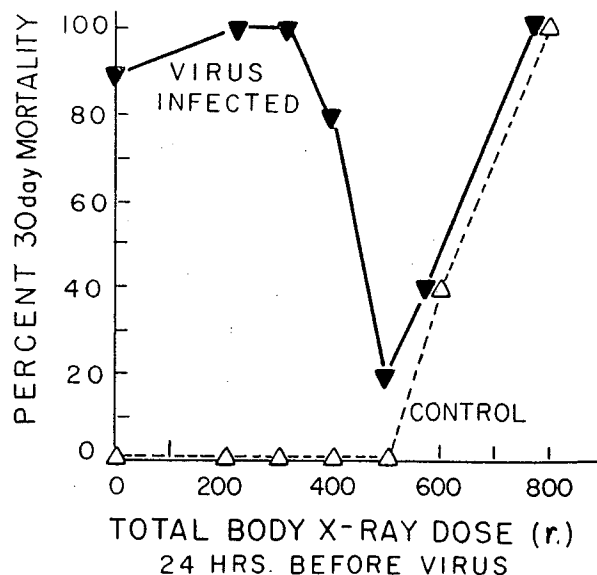


FIGURE 9. The effects of different doses of X-ray given 24 hr before ic LCM virus. Mortality is measured as the percentage total animals which died (out of 10), during a 30-day period after X-irradiation. Control animals received normal mouse brain ic instead of virus.

was not due to any inhibition of virus multiplication by the X-ray since titration curves of virus levels in the blood and brain showed the titers of the X-rayed mice to be comparable to those of a control group; if anything, those of the X-ray group were higher (Fig. 10). The X-rayed animals remained free from disease in spite of high virus titers and appeared to be exactly the same as neonatal PTI animals of the same age; long-term follow up was not done. To correlate the protective effect

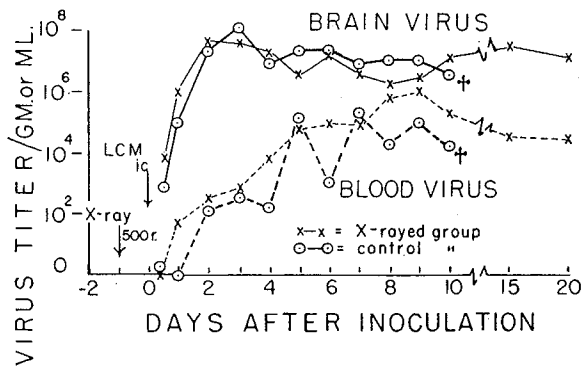


FIGURE 10. The effect of 500 r X-irradiation given 24 hr before  $10^4$  LD<sub>50</sub> LCM virus ic, upon brain and blood virus titer. † = all mice dead.

with other effects of the X-irradiation, 2 individuals of a group of mice were sacrificed daily and their heart blood was sampled for total leucocyte counts. Results of these counts on animals receiving X-ray, virus, or both are shown in Fig. 11. The dose of X-rays used caused a severe leucopenia, to a low value of about 1000 cells/cmm in the controls, and about half this in the virus-infected group. The leucopenia lasted for about 10 days in the control group which showed reasonable agreement with the duration of protection afforded by 500 r X-rays (6–8 days) (Fig. 12) as measured by per cent mortality during 28 days following virus inoculation at different intervals after the X-irradiation.

The relative absence of white cells from the blood and concomitant freedom from disease in the irradiated mice suggested that it was the lymphocytic infiltration of the tissues which actually caused the clinical signs of LCM. This suspicion was further borne out by histological study of the mice sacrificed for the leucocyte counts. It was found that the X-irradiation itself caused very little change in control uninfected mice except the expected hematopoietic hypoplasia, and that, in

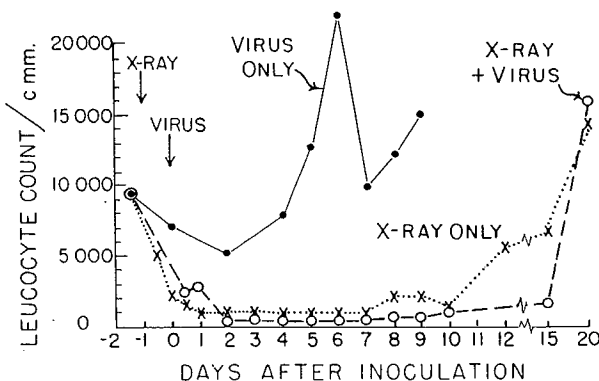


FIGURE 11. Heart-blood leucocyte counts on groups of mice receiving either 500 r X-ray,  $10^4$  LD<sub>50</sub> virus ic, or both of these. Each point represents the average of duplicate counts on each of 2 mice.

the mice receiving LCM only, there was the usual widespread lymphocytic infiltration; however, in the group which was both irradiated and infected, only the X-ray hypoplastic changes were found; all histological evidence of virus infection including lymphocytic infiltration and hepatic necrosis had been eliminated in spite of normal virus multiplication (Collins, Weigand and Hotchin, 1961). Figure 13 shows stained sections of control virus-infected liver showing extensive infiltration with focal granulomatous inflammation, and virus-infected liver with prior irradiation showing an entirely normal appearance; in both cases, the animals were sacrificed 7 days after inoculation. It is noteworthy that a definite sparing effect was also found by treating infected mice with large doses of cortisone

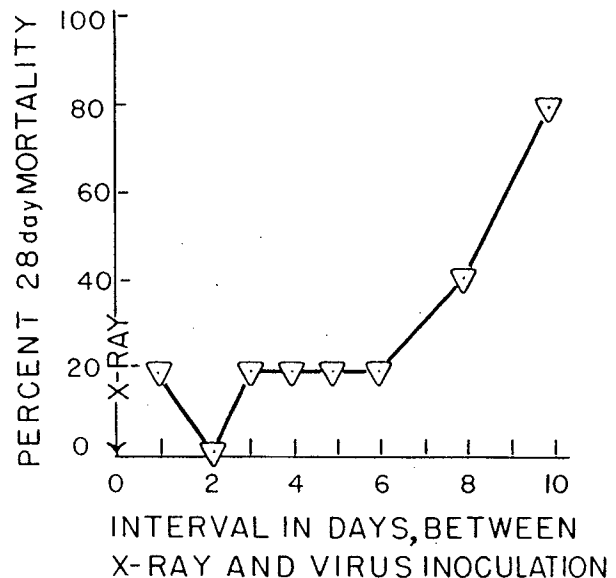


FIGURE 12. The relationship between per cent 28-day mortality and the interval between 500 r X-irradiation and subsequent ic inoculation with  $10^4$  LD<sub>50</sub> LCM virus.

(Hotchin and Cinits, 1958). These results confirm Rowe's (1956) observation and offer strong circumstantial evidence that both the pathological changes and clinical disease following LCM inoculation of adult mice occur as a direct result of the immune response, probably the cellular component of this, and that the disease is eliminated if the immune response is suppressed. This end result is a state of immunological tolerance or paralysis with continued virus multiplication, comparable at least temporarily to the PTI mice obtained by neonatal infection.

#### THE EFFECT OF AMETHOPTERIN

The anti-folic acid agent amethopterin was reported by Haas and Stewart (1956) to exert a sparing effect upon LCM infected mice without

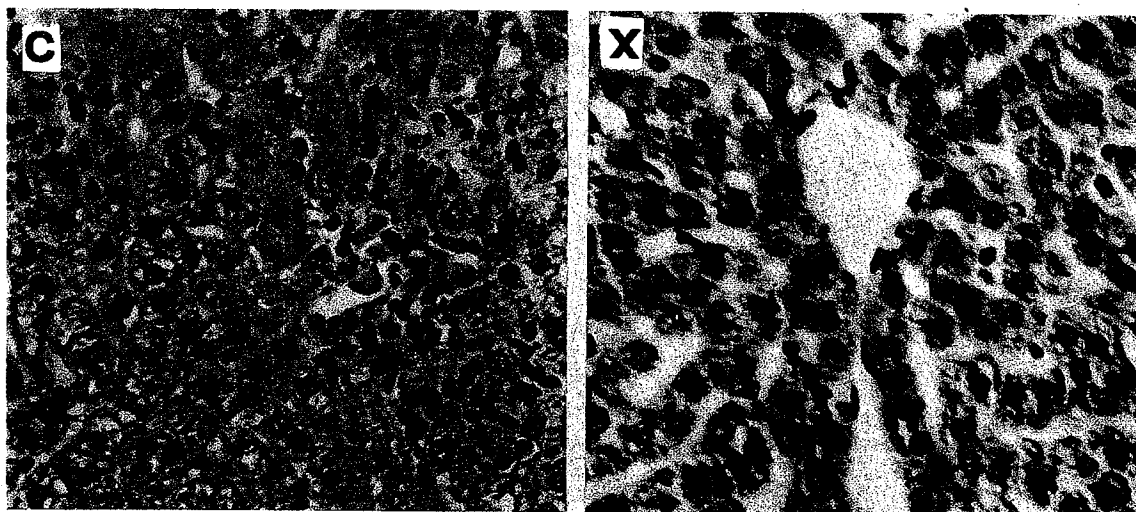


FIGURE 13. Typical areas of liver from mice inoculated ic 7 days previously with  $10^4$  LD<sub>50</sub> LCM virus. C = control mouse receiving virus only. X = mouse which received 500 r X-rays 24 hr prior to  $10^4$  LD<sub>50</sub> LCM virus ic.

eliminating the viremia, and by Lerner and Haas (1958) to prevent the normal histological picture of lymphocytic infiltration. These results were believed by Levy and Haas (1958) to occur through some biochemical effect although a secondary effect on the host was considered; it now appears that a tolerance inducing mechanism is involved. In my laboratory amethopterin has been used as a tool, chiefly by Dr. James Barlow, to investigate the mechanism of tolerance induction by LCM virus. By using even a single injection of amethopterin in the correct dosage (8 mg/kg), and at a precise moment (100 hours after ic inoculation) during the incubation period of the LCM disease (Barlow and

Hotchin, 1962a), the host response can be so modified that instead of complete mortality, 80% of the animals suffer only mild temporary illness and then recover with persistent tolerant infection (Fig. 14). There seems to be no doubt but that this anti-leukemic drug is able to impair the immune mechanism of the host at a crucial moment during the developing immune response so that active immunity is inhibited and complete tolerance results. In later experiments the critical requirements of single drug administration were obviated by giving 2 doses (on day 4 and day 6, 4 mg/kg) following virus; experiments showed that all animals so treated were immune to repeated virus challenge 30, 60, and 90 days after infection, and virus titrations of the brain and blood of random mice showed that this immunity was of the tolerant type (Fig. 15) since high virus levels persisted

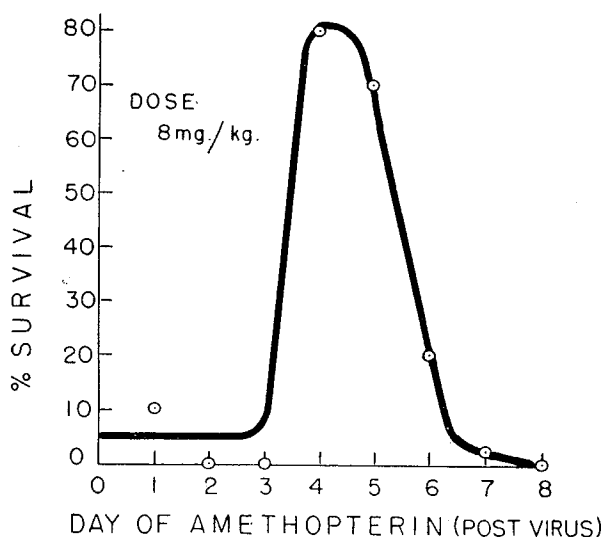


FIGURE 14. The relationship between per cent survival and day of administration of 8 mg/kg amethopterin, after ic inoculation of approximately 100 LD<sub>50</sub> LCM virus, to adult mice. This dose of drug had no visible effect upon uninoculated mice.

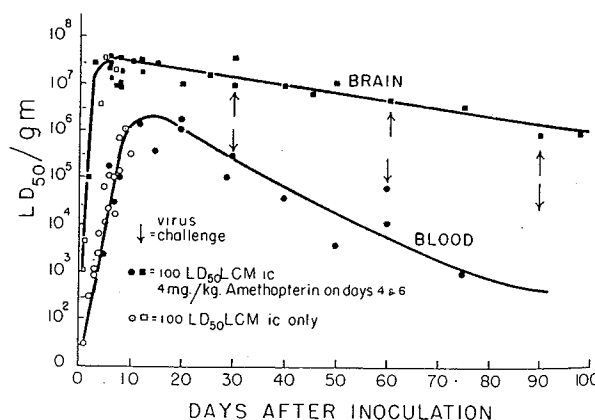


FIGURE 15. Virus titer in brain and blood of mice with and without amethopterin treatment, 4 mg/kg on days 4 and 6. The untreated mice died, but treated ones showed only slight temporary illness. Arrows show times of ic virus challenge.

throughout the duration of the experiment, particularly in the brain; spleen and liver titers fell between those of brain and blood, the spleen being the higher. After the temporary illness between days 5 and 15, the mice appeared perfectly well throughout the 90-day experimental period and it was concluded that they were essentially the same as the PTI mice obtained by neonatal inoculation. The clinical condition of the animals is well shown by their average weight curve (Fig. 16); although

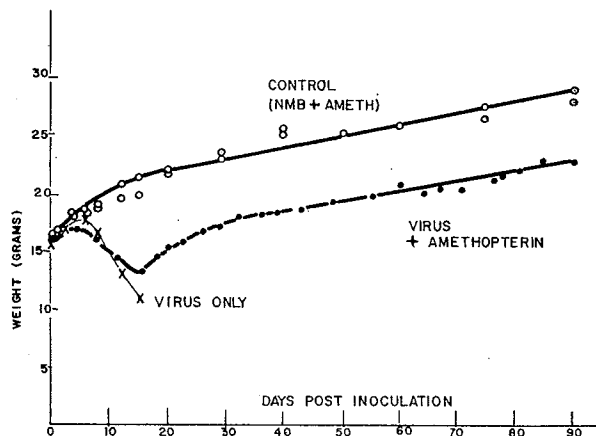


FIGURE 16. The weight curves of mice inoculated with: 1. normal mouse brain and amethopterin. 2. LCM virus plus amethopterin. 3. LCM virus only. Routes and dosage were the same as in Fig. 15.

they suffer only temporary weight loss at the same rate as the virus controls, which all died, the PTI animals never regain their normal weight, their average remaining about 5 g below the control group; this situation is identical to that subsequently found (see below) during long-term study of neonatal PTI mice. The effect of amethopterin as a tolerance-inducing agent is clearly different from that of X-irradiation; no marked or lasting effect on leucocytosis could be detected and the impression gained is that the drug impairs the

"learning ability" of the immune system during a critical stage of its response to the LCM antigen. The effect again suggests, as in the runt phase of neonatally infected mice, a negative feedback mechanism with complete inhibition of any further response toward active immunity, the net result being tolerance. Recent work by Barlow (1962a) indicates that the critical timing of the drug administration is governed by the virus dosage, i.e., drug concentration must be at its peak coincidentally with peak virus titer, for tolerance to result. The effect of this drug upon the host response to virus infection suggests the possibility that similar immune suppression during the incubation period of human virus disease might cause tolerance induction if comparable types of virus exist in man.

#### THE EFFECT OF SUBCUTANEOUS PRE-INOCULATION ON VIRUS CHALLENGE

The results obtained with neonatal inoculation, X-rays, and amethopterin showed that depression of immune response to LCM tended to eliminate disease and supported the concept of immune conflict as the main cause of disease. Traub's (1938) observation that ic challenge of mice with waning immunity resulted in accelerated death led Burnet and Fenner (1949) to suggest that this effect was due to a sensitivity reaction, and Lyon (1940), Haas (1954), and Rowe (1954) all noted similar effects. This question was reinvestigated (Seamer et al., 1962) to test the point of view that increasing sensitivity develops to ic challenge during short intervals after sc inoculation; the main results of the study are shown in Table 2 and diagrammatically in Fig. 17. Forty mice were used for each group and all of the mice which were inoculated ic with  $10^{2.3}$  LD<sub>50</sub> LCM on day 0 were dead by the eighth day. Only 2 of a similar group given this dose sc died, these deaths being at the twelfth

TABLE 2. THE EFFECT OF SC INOCULATION OF MICE WITH  $10^{2.3}$  LD<sub>50</sub> LCM VIRUS (UBC STRAIN) UPON SUBSEQUENT IC CHALLENGE WITH A SIMILAR DOSE

Day of virus inoculation		Deaths <sup>a</sup>	Mortality %	Mean day of death <sup>b</sup>	Standard deviation
SC	IC				
0	—	2/40	5	12.5	±2.12
—	0	40/40	100	8.0	±0.70
0	1	40/40	100	8.4	±0.78
0	2	40/40	100	8.0	±0.48
0	3	40/40	100	8.2	±0.41
0	4	35/40	88	8.9	±0.59
0	5	7/39	18	10.7	±2.43
0	7	4/37	11	12.3	±2.50
0	50	3/37	8	59.7	±3.51

<sup>a</sup> Numerator = number dead; denominator = number inoculated.

<sup>b</sup> Measured from first inoculation.



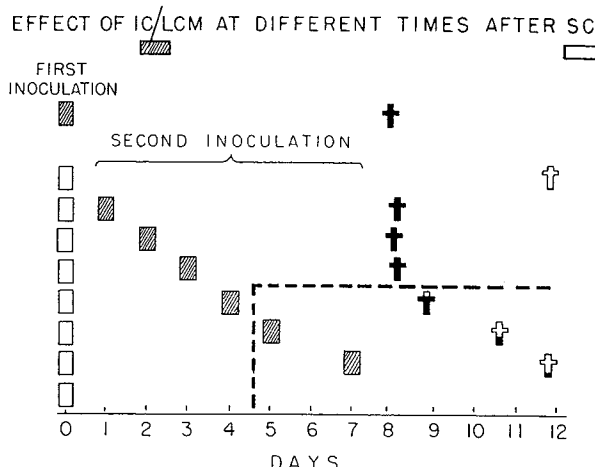


FIGURE 17. Diagram showing the effect of pre-inoculation with virus given sc, upon ic challenge at different intervals later; sc inoculation is shown as an open box and ic as a shaded box. Deaths are represented as a cross, filled in proportion to the number of dead animals; 40 mice were used in each group.

day. Groups given a first inoculation sc on day 0 and the same dose ic on days 1, 2, or 3, all showed 100% mortality at day 8, indicating that the sc inoculum had sensitized the animals and had set the time of death. Challenge on or after the fourth day showed decreasing mortality and progressively later times of death indicating that at day 4 sensitivity passed into immunity with resistance to challenge. Such mice were able to suppress the challenge virus and remained actively immune with the development of complement-fixing antibody. These experiments showed that presensitization of the immune mechanism of the animal to the virus caused an accelerated response to ic challenge. If immunity was incomplete when the high virus titer of ic inoculation occurred (see Figs. 10 and 15), the animals died, whereas if the immunity had reached high levels before peak virus titer, the infection was rapidly suppressed. The deaths of acute LCM disease in adult mice may thus be due—as Barlow has suggested (Barlow, 1962b)—to a strong immune response occurring in a phase of antigen excess causing an anaphylactic type of shock, or may be a consequence of a marked cellular immune response occurring when the bulk of the animals' cells exhibited LCM surface antigen; in the latter case, LCM disease can be regarded as a generalized delayed hypersensitivity reaction.

An unusual effect was noticed after mouse foot-pad inoculation with the virus, consisting of edema of the foot and leg about 8 days later (Hotchin, 1962a). This response is less evident in young mice than in older ones and is prevented by pre-irradiation of the whole mouse prior to virus inoculation. Histological study of the affected mice indicates a severe obliterative lesion of the local lymph node

which may follow a hypersensitivity reaction in the local node and cause the edema by obstruction. The observation focuses attention on the role of the lymph node in the early stages of infection with this virus. Although no evidence of cross-reaction or contamination with ectromelia virus could be found (Hotchin, 1962a), this result suggests similarities with the pathogenesis of ectromelia described by Fenner (1948), who suggested an allergic component in the host response to that virus.

### THE EFFECT OF EPERYTHROZON COCCOIDES

The harmless red blood cell parasite of mice, *E. coccoides*, is known to convert an inapparent mouse hepatitis virus (MHV) infection of mice to a lethal one (Gledhill, Dick, and Niven, 1955). Owing to the prominence of hepatitis in LCM virus infection, the effect of *E. coccoides* on this virus was investigated (Seamer et al., 1961). It was found that an increase in susceptibility occurred in *E. coccoides* infected mice with respect to sc inoculated LCM virus, in a similar manner to the MHV effect. The mechanism of the effect is believed to involve the reticuloendothelial cells.

### THE EFFECT OF BACTERIAL ENDOTOXIN

Tests with this agent have shown (Barlow and Hotchin, 1962b) that mice infected with LCM virus develop considerable sensitivity to *Escherichia coli* endotoxin during the incubation period following ic inoculation with LCM as judged by the lowering of the minimum lethal doses (Table 3). This sensitivity increased rapidly as the incubation period

TABLE 3. THE EFFECT OF ENDOTOXIN (IP) ON MICE INOCULATED IC WITH  $6 \times 10^5$  LD<sub>50</sub> LCM VIRUS

Day of endotoxin injection (post virus)	Endotoxin MLD ( $\mu$ g)
No virus	>1800
1	300
2	300
3	300
4	75
5	<5
6	<0.63

progressed to signs of sickness on about the fifth day following virus infection. By the sixth day, following a large dose of virus ( $6 \times 10^5$  LD<sub>50</sub>), sensitivity to endotoxin was approximately 3000 times greater than normal; smaller virus inocula ( $10^2$  LD<sub>50</sub>) gave the same effect but this sensitivity was reached about 1 day later. In these experiments the most striking clinical event was the fact that

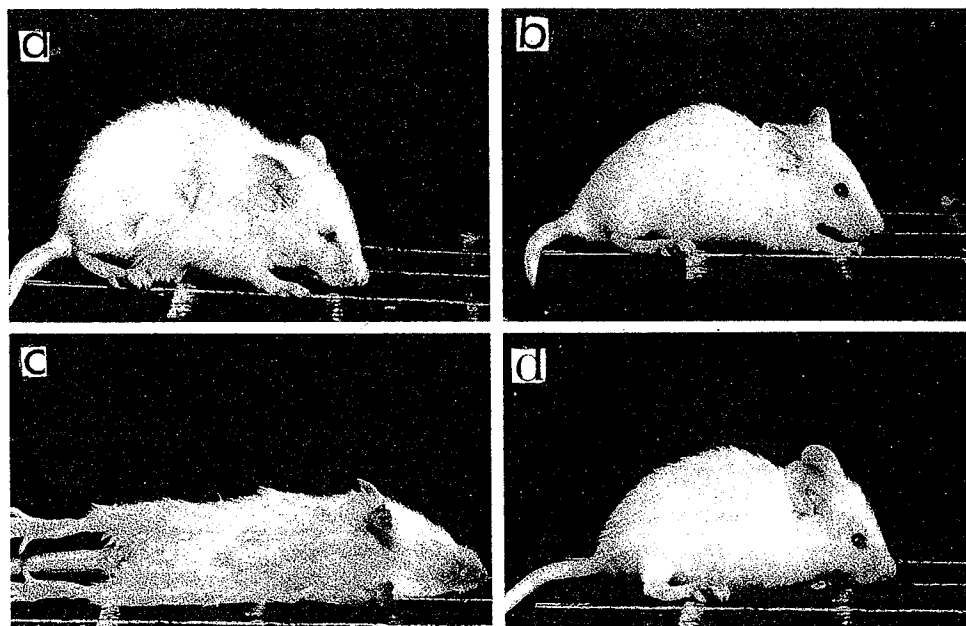


FIGURE 18. The effect of endotoxin on mice after ic inoculation with  $10^5$  LD<sub>50</sub> LCM virus. All mice were photographed on day 3. (a) mouse received 150  $\mu$ g endotoxin on day 0, and virus day 0. (b) mouse received virus day 0 but no endotoxin. (c) mouse received virus day 0 and 300  $\mu$ g endotoxin day 1. (d) mouse received no virus, but normal mouse brain ic on day 0, also 300  $\mu$ g endotoxin day 1.

mice dying within 24 hours of endotoxin injection presented exactly the same signs as mice dying from ic LCM virus inoculation, i.e., they showed hunched posture, extremely ruffled fur, blepharitis, and convulsions on spinning; this was true even when the whole sequence of endotoxin injection and death occurred during the first 30 hours after ic LCM inoculation. Figure 18 shows the effects of virus and endotoxin; photographs were taken on day 3 relative to virus. Virus alone caused no clinical effect (b); 300  $\mu$ g of endotoxin plus normal mouse brain suspension caused only slight ruffling (d); 150  $\mu$ g of endotoxin plus virus on day 0 caused marked sickness (a); and virus on day 0 plus 300  $\mu$ g endotoxin on day 1 caused fatal convulsions on spinning (c). This picture of endotoxin-LCM disease and death is exactly the same as that described by Suter and Kirsanow (1961) following endotoxin injection of mice suffering from infections with mycobacteria (Suter, 1962). The clinical disease appears to be the same in the case of (1) acute LCM death occurring on about the eighth day or (2) acute death caused by hypersensitivity to endotoxin resulting from mycobacterial or (3) intracerebral LCM infection as early as the second day of the incubation period, and later following sc inoculation. It seems most probable, therefore, that the 3 cases involve a common mechanism. Suter and Kirsanow (1961) have emphasized the development of increased sensitivity to endotoxin during infections in various animals, including

murine hepatitis (Gledhill, 1958), *E. coccoides* (Gledhill and Niven, 1957); and Stetson (1959) has suggested that the common feature shared by these reactions is the mechanism of delayed hypersensitivity. While a final statement cannot be made at the present time, it does appear that this view is at least close to the facts and, as previously suggested (Hotchin and Weigand, 1961a), the acute disease of LCM virus is actually an acute hypersensitivity reaction to a replicating intra- and extracellular antigen. In addition, this reaction may be augmented by a concomitant increase in sensitivity to endogenous host endotoxin.

#### THE EFFECT OF LEUKERAN

Mention should be made of the endotoxin-like activity of the radiomimetic nitrogen mustard derivative chlorambucil (Leukeran), which has been found by Barlow (1962c) to precipitate an acute "endotoxin shock" syndrome during the incubation period after ic LCM inoculation of Albany (but not Swiss) strain albino mice. This drug exerts a pronounced leucopenic effect (particularly on lymphocytes) comparable to X-irradiation (Elson, 1958); it had no visible clinical effect upon normal mouse brain inoculated control mice in the dosage employed (32 mg/kg) but caused a 95% reduction in circulating leucocytes within 48 hours. Table 4 shows the effect of single ip injections of this dose upon mice at various intervals after infection with

different amounts of LCM; mortality which occurred during the 24 hours after inoculation was measured and found to be proportional to virus dose and to increase with time of drug administration after infection. Some 30% of mice finally

TABLE 4. THE EFFECT OF IC VIRUS DOSE ON RESPONSE OF MICE TO IP LEUKERAN (32 mg/kg)

Virus MLD <sub>50</sub>	24-hr-mortality. % after Leukeran on day:		
	2	4	6
10 <sup>6</sup>	80	100	100
10 <sup>4</sup>	20	90	100
10 <sup>3</sup>	0	80	100
10 <sup>2</sup>	0	30	60

recovered after 3-4 weeks of chronic illness (compared to 100% mortality in virus-only controls) and these all proved to be PTI comparable to amethopterin-treated mice. It thus seems that this effect has some of the attributes of both endotoxin and amethopterin. Whether endotoxin pretreatment can protect against LCM, possibly giving PTI animals, has not been ascertained. It is tempting to speculate that Leukeran may, like endotoxin, act on the cellular response which has developed in response to virus infection, presumably the lymphocytic infiltrate or conceivably a progressive change in the reticuloendothelial system (RES).

#### THE EFFECT OF DIFFERENT VIRUS STRAINS

When early mouse brain passages of LCM virus were used to inoculate newborn mice as described earlier, mortality was very low and surviving mice were all tolerant. However, when later mouse brain passes were used, the tolerance-inducing capacity had apparently been lost and all the newborn mice died some days later with the usual signs of LCM. Further study of this effect showed that virus stocks maintained by ip passage and liver harvest retained full tolerance-inducing capacity and even improved in this respect (Hotchin, Benson, and Seamer, 1962). Furthermore, passage of the "brain" virus 6 times by ip route restored its tolerance and, similarly, brain passage of the "liver" strain produced a virus which failed to induce tolerance. Strains which possessed the tolerance-inducing capacity in newborn mice were called "docile" and the brain virus which seemed more active in causing the immunological conflict was called "aggressive." There appears to be a parallel here between docility and viscerotropism, and between aggressiveness and "neurotropism"; the latter seems to be a term of doubtful validity

when applied to LCM except with reference to passage route, since the "neurotropic" effects (excitability and convulsions) can be obtained in several other ways, such as ip inoculation with LCM, ip endotoxin injection during LCM incubation period, murine anaphylactic shock (Weiser, Golub, and Hamre, 1941) and also after ic inoculation of large amounts of influenza virus (Henle and Henle, 1946). When both docile and aggressive types of virus are inoculated together in equally high doses into newborn mice, tolerance results, indicating that the docile virus interferes with the multiplication of the aggressive strain (Hotchin and Benson, 1962). This appears to be similar to the interference described by Rowe (1954) whereby a viscerotropic LCM virus strain interfered with exudate production in adult mice by a neurotropic strain. In view of the importance of the passage history in determining the character of the LCM virus, strains were named according to their passage history; thus, M/B<sub>7</sub> = a strain resulting from 7 passages in mouse brain; M/B<sub>6</sub>L<sub>10</sub> = a strain resulting from 6 brain and 10 liver (ip) passes. Most of the preceding experiments were performed with M/B<sub>2</sub> or M/B<sub>7</sub> strains.

#### THE EFFECT OF DIFFERENT HOST STRAINS

Different responses occur in different mouse strains as a result of LCM infection. However, the various minor differences shown by the 2 mouse strains, "Albany" and "Swiss", used in these investigations (Hotchin and Weigand, 1961a) do not affect the conclusions and therefore will not be discussed in detail. An interesting result of the apparent lower reactivity of the Swiss mice is that very high doses of docile virus (c. 10<sup>6</sup> LD<sub>50</sub>) by the ic route can result in less severe disease with survival of the animal while a lower dose of virus would have killed the mouse. This effect, which has been noticed by others with LCM virus, apparently results from the induction of immunological paralysis or tolerance by a very high dose of virus in a susceptible mouse strain.

#### THE MECHANISM OF ACUTE LCM VIRUS DISEASE IN MICE

It is now quite clear that the murine disease process following inoculation of LCM virus is a plastic phenomenon, the form of which can be varied by many factors, making the disease more or less severe. These interrelationships are summarized diagrammatically in Fig. 19. The factors can be listed under 2 groups (Table 5) as disease or tolerance-inducing agents; most of them are

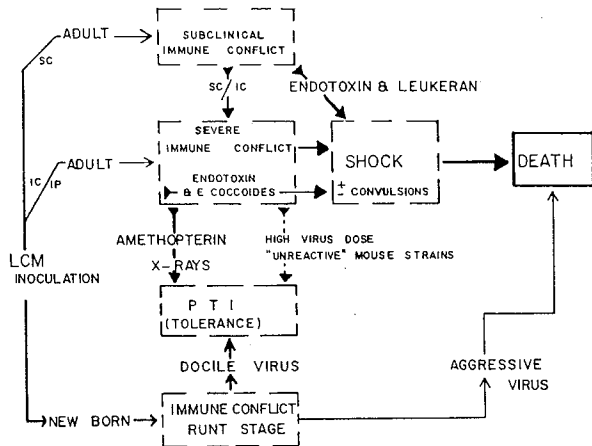


FIGURE 19. The interrelationship of pathogenic mechanisms of LCM disease.

known to produce (1) depression of the immune response, (2) stimulation of immunity, or (3) an anaphylactic shock or generalized Schwartzman phenomenon type of response in animals already in a state of hypersensitivity to another antigen.

TABLE 5. FACTORS AFFECTING LCM DISEASE

Tolerance inducing	Disease inducing
* X-rays	† <i>E. coccoides</i>
* Amethopterin	† Endotoxin
* Leukeran	Leukeran
* Cortisone	Δ SC sensitization
	(+ partial immunity)
* Immaturity (newborn)	Δ Maturity (older age)
* Very high inoculum dose (IC)	Low inoculum dose (IC)
Docile virus	Aggressive virus

\* = Immunity-depressing agents.

† = Shock producing in hypersensitive animals.

Δ = Immunity-stimulating agents.

The latter category, as suggested under endotoxin effects, is of crucial importance in the understanding of this virus disease and possible others, e.g., mouse hepatitis virus infection in which activation by *E. coccoides* occurs and in which certain neurotropic strains can cause a convulsive type of death very similar to LCM convulsive deaths (Gledhill, 1961). The more severe form of disease resulting from ic inoculation with LCM virus, and possibly with MHV, too, appears to be due to the production by this route of sufficiently large amounts of viral antigen and "immune response" to precipitate reactions of which convulsions are the terminal stage. Thus LCM virus disease seems to involve the development of immunological hypersensitivity, which of itself or as a result of additional stress such as that imposed by endotoxin or perhaps by antibody-antigen ratios causing anaphylaxis (as described by Tokuda and Weiser, 1958) produces in certain mouse strains the sequence of incompletely

understood changes which result in the symptoms and death typical of shock produced by several types of agent. In all of these, common pathways are conceivably involved as suggested by Weil and Spink (1956) for mice suffering either from hypersensitivity to an antigen or from endotoxin effects. These clinical signs and alternative causes are summarized in Table 6.

TABLE 6. CONDITIONS CAUSING "LCM" SIGNS IN MICE

Clinical Signs: Ruffled fur, blepharitis or facial edema, hunched posture, convulsions and death

Causes	Reference
LCM virus infection	Weiser, Golub and Hamre, 1941
Anaphylactic shock*	Wheeler, Brandon and Petrenco, 1950
Generalized Schwartzman phenomenon	Stetson, 1961
Endotoxin	
+ hypersensitivity	Suter, 1962
Mouse hepatitis, neurotropic strain	Gledhill, 1961
Influenza virus ic	Henle and Henle, 1946

\* In certain mouse strains.

The basic events underlying LCM pathogenesis thus begin to emerge as dual alternative pathways of the host immune response depending on whether this is positive (active immunity) or negative (tolerance); these are summarized diagrammatically in Fig. 20. Shortly after ic infection there is presumably activity by the RES resulting from the

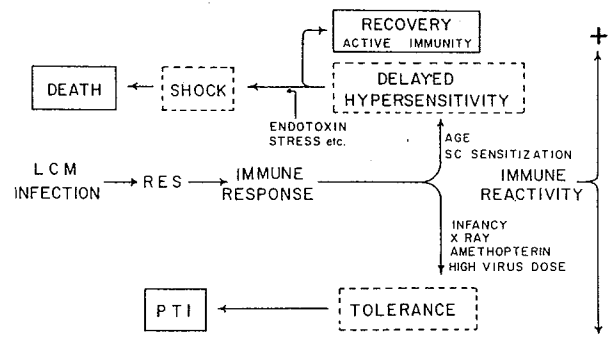


FIGURE 20. The main alternative pathways of LCM pathogenesis and factors influencing them.

invasion of inoculated virus into the blood stream, and possibly causing the increased sensitivity to endotoxin which occurs as early as the first day after infection. Thereafter the immune response occurs and current views indicate that the cellular component of this or the steadily increasing activity of the RES consequent on rising blood virus titer results in the steady rise in endotoxin sensitivity. During this period the immune response may be increased or decreased by appropriate factors and,

when significant response is present, the animal exhibits proportional hypersensitivity to stress (e.g., endotoxin, Leukeran, "spinning"), exhibiting the typical signs culminating in convulsions and death. After ic inoculation, apparently the hypersensitivity is severe enough to follow this pattern to completion in the absence of additional stress agents, by a process comparable to severe generalized delayed hypersensitivity to the replicating viral antigen. At the other end of the scale of host immune reactivity, a negative response constitutes tolerance and results in the PTI state.

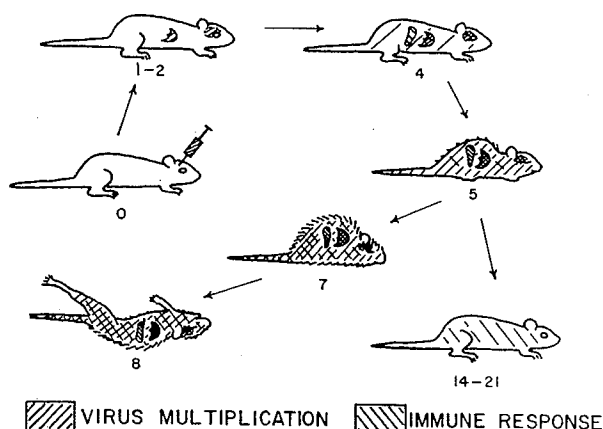


FIGURE 21. Diagram of a proposed immunological pathogenesis of LCM virus disease; acute virus-induced immune disease. Figures show time in days after ic inoculation (represented by syringe). Virus multiplication is shown in brain and in 1 representative organ (liver) and later throughout the mouse. As the immune response begins to be evident (shown in the spleen, day 4) and becomes generalized, the animal is increasingly sick (days 5-8) from the immunological conflict, culminating in severe shock and death. Virus suppression and active immunity following sc injection are shown for days 14-21.

In the case of a positive immune response (as opposed to tolerance), these concepts can be visualized as an immunological battle, in a form which may have application to other virus-host systems (Fig. 21). The disease occurs as 2 simultaneous processes, those of host and of virus; the latter undergoes rapid local intracellular reduplication (e.g., in brain and liver) with production of antigen and steady rise of blood virus titer. The nature and location of the antigen deserve much more study. It has long been known that much soluble antigen accumulates throughout the mouse, but whether there also occurs a phenotypic mutation or transformation of the infected cell surface as in the case of myxovirus (Hotchin et al., 1958) or there is production of another new accessible antigen of the infected cell, as suggested by Habel (1962) for polyoma virus, is not known. Preliminary fluorescent antibody studies in my laboratory indicate a surface concentration of LCM antigen in infected mouse tissues. Meanwhile the host response involves

the poorly understood mechanisms of development of hypersensitivity to the viral antigen, particularly the delayed form sustained by hyperplastic responses in the spleen and lymph nodes. As the lymphocytic response develops, a generalized invasion of the infected mouse tissues occurs to attack the foreign antigen of the virus transformed cells and a state of generalized delayed hypersensitivity results. This not only may explain the function of the lymphocytic or "round cell" response found on histological examination of LCM-sick animals but also goes much further, suggesting that the main host defense mechanism against this virus infection is by the homograft response, whereby infected "virus-colonized" host tissue is recognized as "foreign" and is promptly and steadily attacked by the lymphocytes in the same manner as skin or organ homograft. In this light the function of the round cell infiltration is seen not merely as a source of local antibody formation but as a curative agent to eliminate the "foreign" antigen of virus infected cells, which are either "cured" or become dead candidates for secondary polymorphonuclear phagocytosis. Such a generalized acute autoimmune attack on a large scale could be expected to cause severe disease, in a form closely related to other severe immunological reactions such as prolonged anaphylactic shock and acute runt disease.

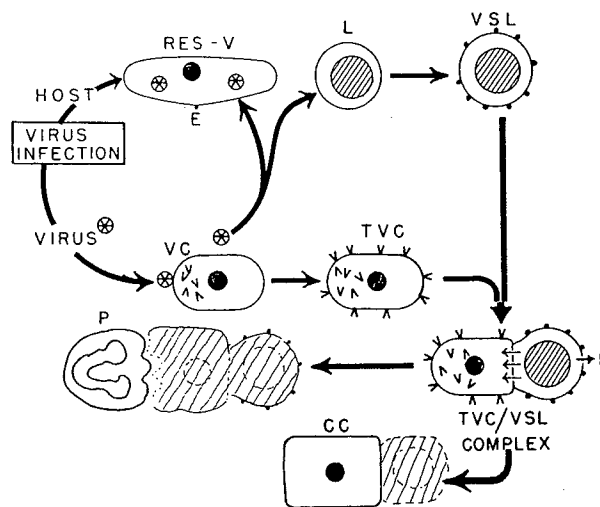


FIGURE 22. Diagrammatic representation of hypothetical LCM host-virus interaction at the cellular level. RES-V = reticuloendothelial system plus phagocytized virus particles, L = lymphocyte, VSL = virus-sensitized lymphocyte, VC = virus infected host cell, TVC = transformed virus infected host cell, P = polymorphonuclear leucocyte, E = possible site of endotoxin effect, CC = "cured" host cell.

These basic concepts can be summarized as a general theory of non-cytocidal virus disease, which is summarized diagrammatically in Fig. 22; virus enters both the RES, possibly sensitizing it to endotoxin, and also enters and infects susceptible

host cells (VC). These are transformed by the virus, as a phenotypic mutation, giving transformed virus infected cells (TVC). At the same time virus antigen activates a cellular immune response whereby lymphocytes (L) become sensitized to virus (VSL); these specifically sensitized lymphocytes become fixed in the virus infected tissues by virtue of a specific affinity for the sensitizing antigen on the "foreign" TVC membrane, thus constituting TVC/VSL complexes. It is postulated that the specific antigen/antibody union between the appropriate surface groupings on the opposed cell membranes of the TVC/VSL causes strains which rupture the membranes at these points, thus allowing escape of the antiviral and possibly cytotoxic contents of the VSL into the TVC with consequent recovery or death of the latter by a process analogous to homograft rejection. The TVC/VSL complex may also be highly sensitive to endotoxin (E), if this could penetrate the VSL (and possibly the RES-V) surface, release of the cell contents might constitute generalized liberation of shock-producing substances. The final outcome of this situation will depend on whether the TVC is "cured" by the VSL, producing a virus-free "cured cell" (CC) or whether it is killed by the attack on its antigenicity and removed by phagocytes (P) as happens in the homograft response. It is postulated that this process is a general one in virus disease pathology and may occur in some infections with viruses ordinarily considered to be cytopathic, e.g., the neuronophagia seen in encephalitis or poliomyelitis may be examples of TVC/VSL complexes resulting from lymphocyte attack.

#### ATTEMPTS TO DEMONSTRATE IN VITRO LYSIS OF LCM VIRUS INFECTED CELLS BY SENSITIZED LEUCOCYTES

Numerous attempts were made to demonstrate a specific ability of lymphocytes from an LCM immune or sick animal to adsorb to and lyse LCM virus infected tissue analogous to the lysis of homologous cells by sensitized lymphocytes in tissue culture described by Rosenau and Moon (1961). All of these experiments failed in their object when mixed mouse embryo tissue, adult mouse kidney, or strain L mouse fibroblasts (which show a partial cytopathic effect with LCM virus) were used. However, some success has recently been obtained by using tissue cultures of testis from PTI adult mice as the target tissue for spleen lymphocytes of normal or LCM immune mice. These results are indicative of a direct effect of lymphocytes upon virus carrying cells. Further work is needed to substantiate this observation, and to test for the ability of sensitized lymphocytes to eliminate virus from the cultures.

#### LONG TERM RESULTS OF PERSISTENT TOLERANT INFECTION WITH LCM VIRUS IN MICE

For a long time it appeared that LCM-PTI mice were no different from normal uninfected controls apart from their carried virus; however, quite recently it has become clear that this is not the case in 2 respects. The PTI animals are apparently highly resistant to tumor induction by polyoma virus, and also they suffer in later life from a severe debilitating disease or group of diseases about which little is yet known but which appear to constitute the manifestations of autoimmune disease. These 2 effects will be considered in turn.

#### THE SPARING EFFECT OF PERSISTENT TOLERANT INFECTION WITH LCM VIRUS UPON TUMOR INDUCTION WITH POLYOMA VIRUS

Since PTI mice constitute an unusual situation in which there is long term generalized virus infection in a host, the possibility existed that there would be interference by the LCM virus with the multiplication of a superinfecting viral agent. In addition, the neonatal type of infection provided a unique opportunity for possible interference with a tumor producing agent, such as polyoma virus, requiring inoculation in very early life. There was also the possibility of another form of interaction due to the induction of immunological tolerance by the LCM virus; it was conceivable that LCM might induce tolerance by damaging the immune cells of the host, in which case tolerance to another virus such as polyoma might be enhanced, with consequent greater tumor incidence. These possibilities were tested by inoculating groups of 100 newborn mice with LCM virus, polyoma virus, or both, the LCM being given on day 1, and the polyoma on day 2, after birth. The results of the experiment (Table 7) showed a tumor incidence of 35% in the polyoma group, within 10 months of inoculation, whereas the mice receiving both viruses showed an incidence of only 4%, a 9-fold difference. The rate of incidence of tumors in the 2 groups is shown in Fig. 23. It is clear that this is a significant result, which is probably an example of viral interference. No tests have been made as yet of the status of the groups with respect to polyoma antibodies, and the interesting question is raised as to whether polyoma virus multiplication is prevented or whether LCM virus infection prevents transformation of cells to the malignant state. Recently Jungeblut (1962) has observed a parallel sparing effect of LCM virus upon transmissible leukemia in guinea pigs, which appears to confirm the similar conclusions of Nadel and Haas (1955, 1956). An interesting feature of Jungeblut's results

TABLE 7. THE INCIDENCE OF MALIGNANT TUMORS IN POLYOMA INJECTED NORMAL AND PTI MICE DURING 10 MONTHS

Inoculum (ip)		No. of mice on day 28	Total no. of tumors	Tumor incidence
Day 1	Day 2			
LCM	—	71	0	0%
—	Polyoma	79	28	35%
LCM	Polyoma	52	2	4%
Normal mouse brain	—	78	0	0%

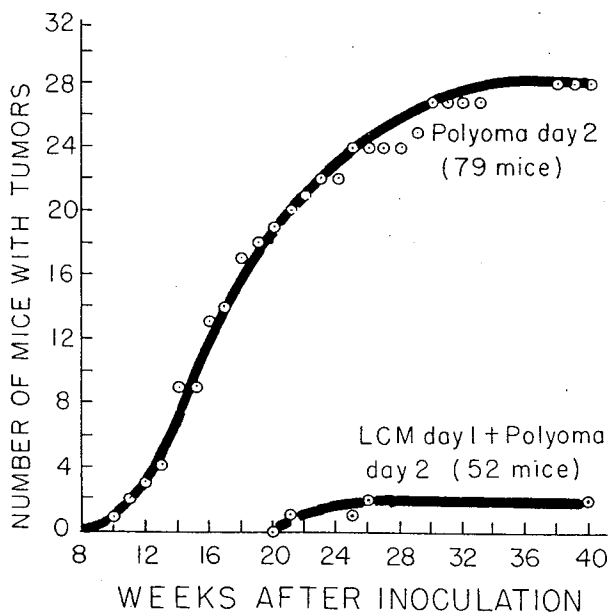


FIGURE 23. Cumulative tumor incidence in mice infected with polyoma virus only on day 2, or LCM virus on day 1 and polyoma virus on day 2, after birth.

is the fact that the doubly-infected guinea pigs do not show signs of LCM disease in spite of the presence of this virus in their blood, suggesting that interaction with the leukemia agent may have rendered them tolerant.

#### THE DEVELOPMENT OF "LATE DISEASE" IN LCM-PTI MICE

Observations of an unusual type of illness developing in stocks of PTI mice led to a retrospective investigation of the disease syndrome occurring in mice approximately 1 year after neonatal inoculation with LCM virus. Owing to the extremely long "incubation period" of the disease, the normal type of experimental approach to its causation was not possible and observations on its development and pathology have of necessity been obtained piece-

meal. Once the immunological etiology of the condition was suspected, several lines of investigation were followed concurrently, which will be presented in turn for the sake of clarity.

#### THE CLINICAL COURSE OF LCM-PTI MICE

Past experience has shown that weight measurements offer the best objective criteria of LCM disease in mice. Weight changes were therefore followed for 7 months in a group of 100 infant mice inoculated ic on the first day after birth with  $6 \times 10^3$  LD<sub>50</sub> LCM virus, and also in a similar group inoculated at the same age with normal mouse brain suspension. The results are shown in Fig. 24. After the first 3 weeks during which runting occurred (as in Fig. 6), the average weight in the virus infected group increased rapidly but failed to reach the values for the control animals, remaining throughout 4-6 gram lower. In spite of the lower weight the mice appeared perfectly healthy and indistinguishable from controls. The mortality of the 2 groups also remained comparable after the initial slight mortality during the runt phase of tolerance induction following virus inoculation;

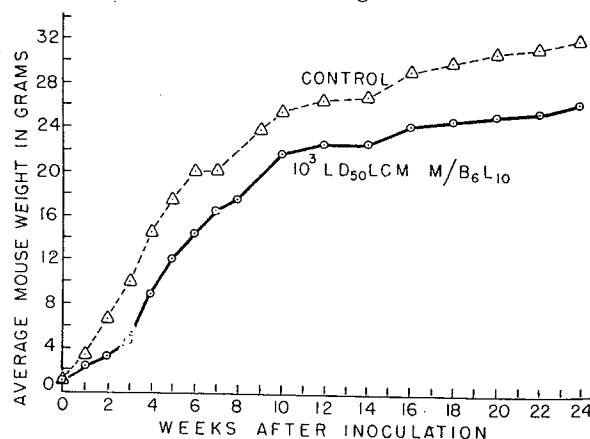


FIGURE 24. Weight curves of normal control (broken line) and LCM virus infected PTI mice (solid line).

FIGURE 25.

- PTI mouse showing moderate late disease, 15 months after neonatal LCM virus inoculation.
- PTI mouse showing severe late disease, 21 months after neonatal LCM virus inoculation.
- Normal uninoculated mouse 19 months after birth.
- A 10-month-old PTI mouse 36 hr after receiving 150  $\mu$ g endotoxin.
- Control 10-month-old PTI mouse.
- PTI/immune parabiotic pair 90 days after operation. PTI shows "late disease" and anemia.
- Same PTI/immune pair as in Fig. 25f, but photographed 20 days later. The PTI mouse died the following day.
- PTI and immune mice from the same groups as mice in Fig. 25f but not joined; photographed at age corresponding to 210 days after joining age of parabionts.
- Normal/immune parabiotic pair showing normal appearance 195 days (6½ months) after joining.

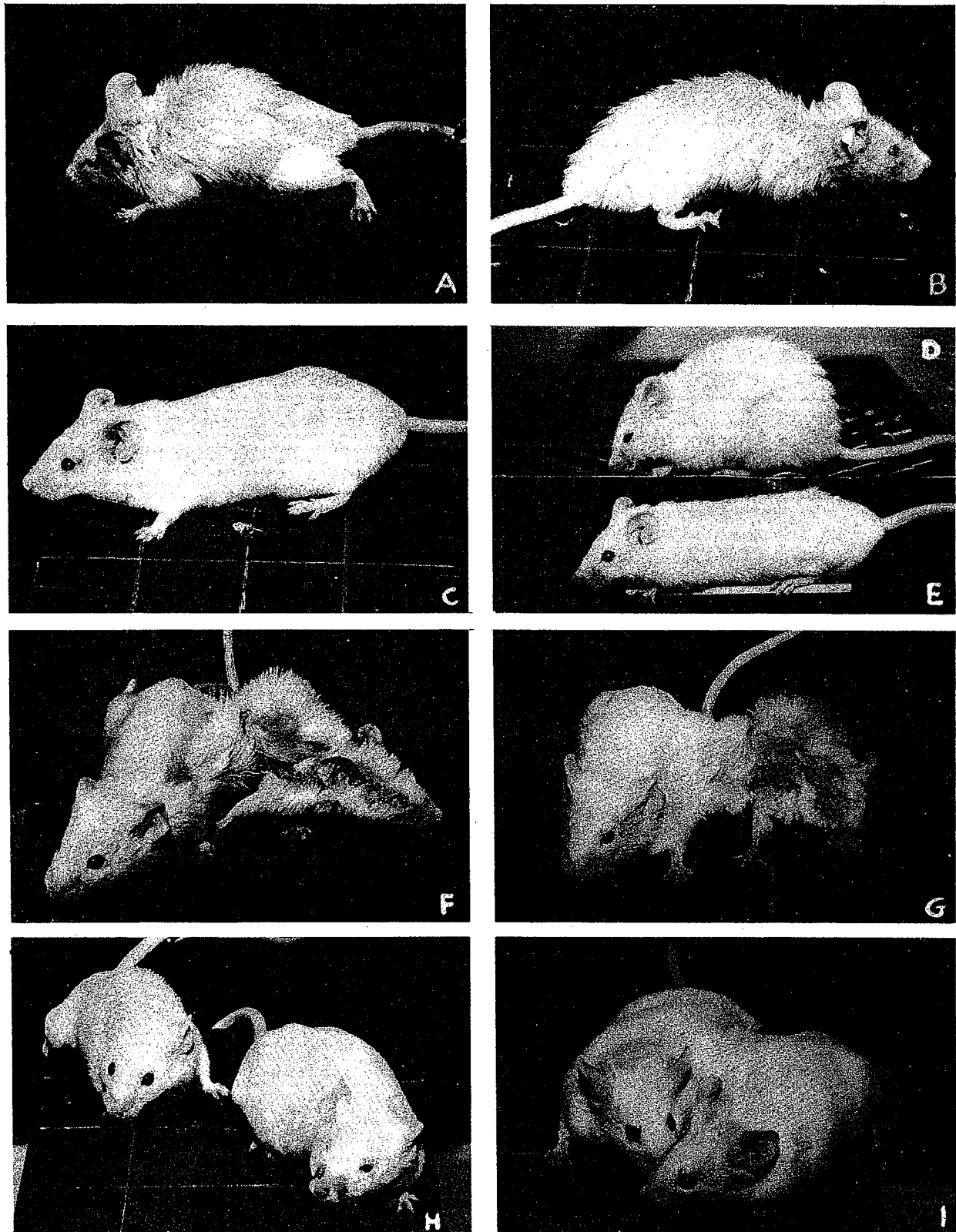


FIGURE 25—See opposite page for figure legend.



thereafter the virus infected group had a very slightly higher rate of death. Unfortunately, this experiment was terminated at 7 months as the groups were believed to be clinically identical. Subsequent observation of older PTI mouse stocks revealed the incidence of a late-onset disease, usually beginning between 10 and 14 months after neonatal inoculation. There was ruffled fur, blepharitis, and hunched posture, resembling early stages of acute LCM disease. The PTI mice, however, did not progress to the convulsive stage but developed degenerative changes of the skin, with marked hair loss and a general dilapidated appearance somewhat reminiscent of the neonatal runt phase. Typical mice in this condition 15 and 21 months after inoculation are shown in Figs. 25a and 25b. A normal uninoculated mouse aged 19 months is shown in Fig. 25c; although comparable numbers of control and PTI mice have been observed over a 2-year period, no "late disease" has been found in the controls, whereas all the PTI animals finally succumbed to this condition and died. During the whole of the life of the PTI animals, virus titrations have shown the presence of fairly high levels in most of the animals examined (Table 8). Histo-

TABLE 8. VIRUS TITERS IN RANDOMLY SELECTED PTI MICE

Months after neonatal inoculation	LD <sub>50</sub> /g		
	Brain	Liver	Blood
13	$9 \times 10^5$	$9 \times 10^6$	$6 \times 10^5$
13	$6 \times 10^5$	$1 \times 10^6$	$1 \times 10^6$
13	$1 \times 10^6$	$3 \times 10^6$	$1 \times 10^5$
13	$3 \times 10^2$	$2 \times 10^2$	NT
20*	$1 \times 10^4$	$1 \times 10^6$	$1 \times 10^5$

\* Showed typical "late disease"

logical changes found in animals suffering from late disease and sacrificed, include splenic pigment deposition (hemosiderin), hepatitis with round cell infiltration, suppurative pyelonephritis with abscess formation, and splenic hyperplasia; most interesting of all, has been the identification by my colleagues, Drs. Victor Tompkins and Doris Collins, of kidney lesions typical of systemic lupus erythematosus (SLE). These lesions have so far only been noticed very recently in one PTI mouse showing late disease and killed 20 months after inoculation. This finding is of extreme interest inasmuch as SLE is a classical example of autoimmune disease, with very clear-cut cytological changes.

#### THE EFFECT OF OTHER AGENTS ON PTI MICE

Several attempts were made in the past (Hotchin and Cinits, 1958) to induce disease in healthy-

looking PTI mice without success. More recently, inoculation with *E. coccoides* was found to induce temporary illness lasting about 1 week in PTI mice 2 to 7 months old without appreciable change in virus titer. These mice were not studied for late after-effects. Endotoxin injection, at a dosage of 5 mg/kg body weight had very little noticeable effect on mice 5 weeks after neonatal inoculation but caused severe illness in 10-month-old PTI animals within 24 hours of administration, progressing to convulsions on spinning in all 5 animals inoculated and to death in the case of 2 of them. Figure 25d shows 1 of the animals in the typical attitude of acute LCM illness; another control 10-month-old PTI mouse from the same experimental group which did not receive endotoxin is shown in Fig. 25e. These mice appear to have the same type of sensitivity to endotoxin as described earlier for ic infected adults challenged during the incubation period. Although the mice showed greater sensitivity to endotoxin shock as they grew older, control uninfected 10-month-old animals showed only minor temporary ruffled fur after receiving the same dose (5 mg/kg) which killed the PTI mice. If endotoxin sensitivity is taken as a sign of a latent hypersensitivity state, it appears that PTI mice pass through an early hypersensitive stage as evidenced by runting, then become immunologically tolerant and lose their hypersensitivity which returns as they get older, ultimately becoming active enough to produce signs of disease and death.

#### THE EFFECT OF INOCULATED GRAFTS OF LYMPHOCYTES UPON PTI MICE

Numerous experiments were made (Hotchin, Barlow, and Sekely, 1961) in attempts to induce disease in LCM-PTI mice by the intravenous (iv) or ip inoculation of varying amounts of viable cell suspensions obtained from lymph nodes, spleen, thymus, and bone marrow of LCM-immune mice. Although some of these grafts (e.g., bone marrow) were shown to be viable as evidenced by capability to prevent death from supralethal X-irradiation, no specific induction of LCM disease in PTI animals was obtained in the 14-day-period during which the animals were observed. In the light of more recent results with parabiotic animals, it appears likely that the cell transfer experiments were concluded too early for positive results to have been seen, since the time taken for disease to occur in suitable parabiotic pairs was much longer than 2 weeks.

#### THE EFFECT OF PARABIOTIC UNION BETWEEN LCM-PTI AND IMMUNE MICE

The effects of large-scale transfer of immune cells and antibody, made possible by coelomic parabiosis,

were studied using mice of different immunological status relative to LCM virus. The operative technic of Martinez et al., (1959) was used to make parabiotic pairs in all combinations between mice which were either uninfected, actively hyperimmune, or PTI with respect to LCM virus. In all but the first experiment, attempts were made to render the group mutually tolerant by inoculating 15 newborn litters iv with pooled spleen and thymus cells obtained by sacrificing 1 mouse from each litter; this procedure was probably unnecessary since the animals were obtained from the same closed colony which had been maintained for 22 years, and were found to accept skin grafts readily from each other. Only mice of the same weight and sex were joined. The main result of these experiments (Hotchin, 1962b; Hotchin and Sekely, 1962) was the induction of "late disease" in the PTI mice joined to the immune with the additional feature of severe anemia. The results in the various other control combinations were of the expected type. The "late disease" signs occurred only in the PTI animal, the immune remained quite normal even for some hours after death of the PTI. The onset and progress of the late disease were more rapid than in single PTI mice but appeared to continue to a more severe degree. A typical PTI-immune parabiotic pair which showed well marked "late disease" and anemia in the PTI are shown in Fig. 25f and also 20 days later in Fig. 25g; the PTI mouse died the following day. For comparison, single PTI and immune mice are shown in Fig. 25h at a time equivalent to 210 days after the date of operation of other animals in the same experimental pool, and in Fig. 25i a normal/immune parabiotic pair in which both retained their healthy appearance for 195 days after joining. Blood counts on the anemic animal showed red cell counts of only 25% the value of the immune animal which was normal. The impression was gained that the blood picture conformed with a hemolytic anemia but no special tests were done to substantiate it. Follow up of 2 PTI/immune pairs with late disease, after surgical separation, showed rapid recovery of the immune and continued deterioration of the PTI, indicating an irreversible change possibly due to colonization of the PTI animal by immune cells from the parabiotic partner.

#### CONCLUSIONS CONCERNING LCM DISEASE MECHANISMS

Evidence has been presented that the state of induced immunological tolerance to carried LCM virus is not an all-or-none state but can exist in various degrees which gradually shift toward increasing immune reactivity as the animal ages.

This results in the gradual onset of what appears to be the same type of delayed hypersensitivity that occurs in the acute stage of LCM disease in adults. If the state of hypersensitivity persists at a high level for a sustained period, damage occurs to the organism as reflected by the early runt phase following neonatal infection, and also the "late disease." The place of anemia in the picture is not clear, but it seems likely that it constitutes a hemolytic component of an autoimmune disease complex. The pathogenic processes of LCM, which appear at certain points to be increasingly complicated, finally turn out to be the manifestations of a fairly simple basic mechanism which can be stated as follows: Virus infection causes immunological reactivity or tolerance and some form of host cell transformation; reactivity, incomplete tolerance, or its breakdown by cumulative stresses allow development of (delayed) hypersensitivity comparable to the homograft response; according to its severity, the host suffers from acute shock or sustained tissue damage constituting chronic autoimmune disease. This concept of LCM-induced acute and chronic autoimmune disease is summarized in Fig. 26. Here the basic immune reactive capacity of the

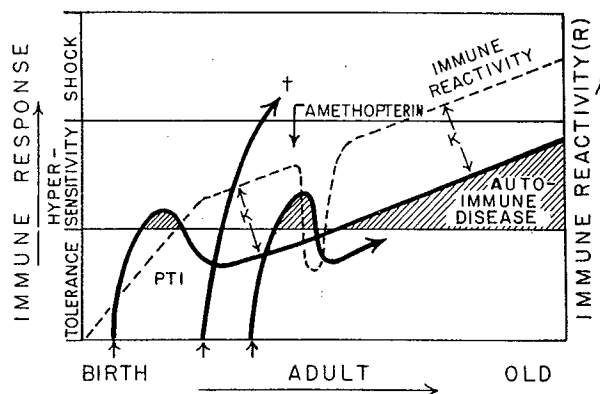


FIGURE 26. Diagram showing the proposed mechanism of LCM virus induced autoimmune disease.

host is represented as a dotted line with respect to the scale on the right, rising steeply in early life and slowly thereafter, and depressed temporarily toward neonatal levels by agents such as amethopterin. The immune response causes tolerance, hypersensitivity, or shock according to its absence or degree of activity and the major effects of infection upon the immune response are shown by the solid lines, according to age at infection as indicated by arrows on the abscissa. The PTI state occurs when the immune response remains in tolerance; the behavior of the system suggests that this is due to an equilibrium reaction between excess of antigen and minute amounts of formed antibody behaving somewhat like a homeostatic

buffer system according to an equation which in its simplest form would be of the type:

$$\frac{[Ag][Ab]}{[AgAb]} = \frac{K}{R}$$

where [Ag] is antigen concentration, [Ab] is antibody concentration and [AgAb] is antigen/antibody complex concentration (both antigen and antibody are considered loosely as either cell bound or soluble); K is a constant for the antigen in question and R is the immune reactivity of the host. Thus

when R is small,  $\frac{K}{R}$  is large and [AgAb] is small with a given rate of Ag production (which is presumably set by the virus). However, as R increases (e.g.,

with age),  $\frac{K}{R}$  decreases and for the same rate of Ag production [AgAb] must increase by Ab production and reaction with Ag, in order to maintain equilibrium; since [AgAb] represents the result of immunological conflict, the end product of increasing R is increasing immunological activity resulting in the tissue damage of autoimmune disease. This theory indicates that immunological tolerance should not be considered as an all-or-none phenomenon but as a dynamic equilibrium, open to quantitative analysis by the standard mathematical procedures of physical chemistry.

#### COMPARISON OF VIRUS-INDUCED AUTO-IMMUNE DISEASE WITH OTHER FORMS OF AUTOIMMUNE DISEASE IN THE MOUSE

Space does not permit a detailed analysis of currently available information on this subject, but certain clear similarities and concurrent trends should be mentioned at this point. The runt disease suffered by neonatal LCM recipients bears a remarkable similarity to the runt disease described by Billingham (1958) and Billingham and Brent (1959) (Fig. 27), produced by injection of newborn mice with spleen cells from a different strain of mice, to which the recipient is tolerant, but which attack their new host by the homograft response. Comparison of Fig. 7 and Fig. 27 illustrates the close similarity of these forms of runting which are indistinguishable. The fact that Billingham and Brent (1959) report that many of their mice recover and gain weight makes an even closer parallel with LCM runt disease and suggests that their mice might also develop late autoimmune disease if observed for periods of 1 year or more. A second striking clinical parallel occurs between acute LCM and the acute "runt disease" of mice described by Oliner, Schwartz and Dameshek (1961), of which a photograph is shown in Fig. 28. Comparison of



FIGURE 27. Two 52-day-old strain A mice from a litter that was injected at birth with 5 million adult C<sub>3</sub>H spleen cells. One mouse is a chronic runt, the other appears unaffected. (Photograph supplied by Dr. R. E. Billingham and reproduced with permission.)

this and Fig. 1 illustrates the similarities between what is here considered as being essentially the same end result but which in Fig. 28 is caused by ip injection of immunologically tolerated splenic cells capable of responding immunologically against the host, to cause acute reactions 28 to 90 days later. This disease picture is also very similar to the shock syndrome caused by endotoxin injection in the incubation period after ic adult LCM inoculation (Fig. 18A) and in previously healthy-looking 10-month-old PTI mice (Fig. 25d). When the appearance of "late disease" is considered, its murine autoimmune character is again strikingly demonstrated by comparison (Fig. 25b) with the late effects of autoimmune disease induced in mice by Oliner et al., (1961) shown in Fig. 29. The fact that Oliner et al. describe moderately severe hemolytic



FIGURE 28. An LAF<sub>1</sub> mouse with runt disease due to ip injection of immunologically competent parental spleen cells. (Reproduced by permission from Oliner et al., 1961, *Blood*, 17: 20-44.)



FIGURE 29. Later stage of runt mouse of the same type as in Fig. 28, showing alopecia and facial edema. (Reproduced by permission from Oliner et al., 1961, *Blood*, 17: 20-44.)

anemia as being a definite part of their graft-induced autoimmune disease of mice makes the anemia found in the parabiotic mice more noteworthy.

The similarities described above are very striking and offer circumstantial evidence that the conditions have a common immunological mechanism; coupled with the known immunological facts, this forms a strong case for the conclusion that the virus induced immune disease is the same autoimmune pathological process as autoimmune runt disease, but with a completely different starting point. The condition is essentially the same as "secondary disease" (van Bekkum, Vos, and Weyzen, 1959) or "homologous disease" (Trentin, 1958) after protective foreign bone marrow injection of supralethally X-irradiated mice. The conclusion that LCM virus can induce autoimmune "late disease" is hard to escape, and, if substantiated by further work, should constitute an important landmark in chronic disease pathogenesis, particularly if the observation of SLE renal lesions is confirmed. In terms of current theories of autoimmune disease causation, as summarized recently by Burnet (1962) there are 3 possible mechanisms: 1) release of inaccessible tissue-specific antigens; 2) malfunction of the normal homeostatic immune mechanism; 3) somatic mutation producing clones of immunologically active cells intrinsically resistant to normal homeostatic control. In respect of these and also similar concepts summarized by Dameshek, Schwartz, and Oliner (1961), the theory of virus induced autoimmune disease propounded here supplies a fourth possibility. This theory differs from previous concepts (Burnet, 1962) of virus triggering since in the present case the virus is regarded as causing a transformation of host target tissue rather than of the host immune mechanism.

## THE IMPLICATION OF THESE RESULTS

The proposed theory of acute and chronic virus induced autoimmune disease has profound implications for human chronic disease etiology in the field of the autoimmune diseases. The murine LCM virus system as a model offers experimental evidence that both early and later life virus infection can cause so-called idiopathic diseases. If there exists a group of similar "docile" human agents, of which serum hepatitis might be one, congenital or even adult infection with these could duplicate the picture of what are now considered to be hereditary chronic diseases of later life. In terms of the biology of host-parasite relationships, the LCM disease pattern enters the realm of what could be called "paragenetics" since congenital and neonatal infection constitutes a form of virus-host symbiosis in which the virus behaves as a replicating entity susceptible to normal intracellular control mechanisms, and although harmless to the cell, causes changes capable of final expression in the form of chronic disease.

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